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THE PHYSICAL AND FUNCTIONAL ANALYSIS OF INTERFERONS.(U)
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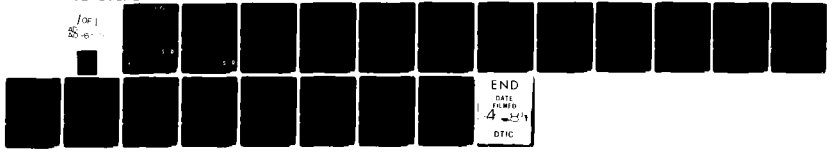

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The objective of these studies has been to analyze the physicochemical, antigenic, and biological properties of both human and murine interferons.

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The Physical and Functional Analysis of Interferons

by

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Trudeau Institute, Inc.
P.O. Box 59
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Final Technical Report January 31, 1981

Workunit No. NR 204-064

Contract N00014-78-C-0610

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The goal of our studies has been to characterize the physicochemical, antigenic, and biological properties of interferons. In the following report, we present the studies which were carried-out during the time of our contract. Some of these studies have been published and those to be published, will acknowledge the financial support of the Office of Naval Research.

Work Accomplished

1. Antibody neutralization studies of homologous and heterologous antiviral activities of Human Fibroblast (HuIFNβ) and Leukocyte (HuIFNα) Interferons.

HuIFNα and HuIFNβ are known to differ both antigenically and in their abilities to exert antiviral activities on cells of other species (1). These properties have proved useful criteria for differentiating between these two HuIFN classes. Studies were undertaken to determine the relationships between the neutralizing titer of specific anti-HuIFN sera for HuIFNs, the quantity of IFN (antigenic mass) reacted and the sensitivity of the assay cell to the test IFN. A quantitative anti-IFN neutralization assay was devised, which measures the degree of neutralization of IFN antiviral activity by virus yield assays. The measurement of IFN neutralization by virus yield is highly sensitive, objective, and allows the construction of IFN neutralization curves. In addition, quantitation of IFN neutralization after a single-cycle virus replication eliminates possible effects of interferon produced by the assay cells in response to the challenge virus during later rounds of viral replication (2).

Two human cell types were used in these studies: The FS-4 strain of diploid fibroblasts and the GM-2504 fibroblast strain, which is trisomic for chromosome 21 and known to be 10-20X more sensitive to HuIFNs than cells disomic for chromosome 21 (3,4). The bovine MDBK cell line was also used because HuIFNα exhibits a high degree of activity on bovine cells, whereas HuIFNβ is only marginally active on these cells (1). Antiviral activity dose-response studies of HuIFNα and HuIFNβ were carried out on the three cell types to determine the quantities of each interferon required to elicit maximum states of virus inhibition. The resulting HuIFNα and HuIFNβ dose-response curves on the three cell types were regularly sigmoidal, had similar slopes, and the differences in the positions of the curves reflected the relative sensitivities of the cells to the two HuIFNs (Figure 1). The GM-2504 cells were 20X more sensitive than FS-4 cells to the antiviral activities of both HuIFNα and HuIFNβ. The HuIFNα was found to exhibit similar activities on both the bovine MDBK and human FS-4 cells, whereas HuIFNβ was 40X less active on the bovine cultures, as compared to its activity on the FS-4 cell cultures.

Anti-HuIFNα serum neutralization curves, obtained for a constant quantity of HuIFNα, on the GM-2504, FS-4 and MDBK cells revealed different patterns of neutralization. The antibody neutralization of HuIFNα on the GM-2504 cultures resulted in a biphasic pattern of neutralization (Figure 2). At the lowest dilution of antibody (1/32), there was complete neutralization of antiviral activity, however, at the next three successive antibody dilutions, there was only partial neutralization and this was reflected as a plateau in the curve at 2 logs (base 10) of virus inhibition. A similar plateau occurred on the FS-4 cells, but was less

pronounced at 0.75 logs of virus inhibition, and no such plateau was observed in the MDBK cells. Previous studies of ours (5) demonstrated the existence of low levels ($\leq$1%) of HuIFNβ in HuIFNα preparations. The plateaus in the anti-HuIFNα neutralization curves for HuIFNα on the GM-2504 and FS-4 cultures were determined to be due to HuIFNβ by reacting a constant quantity of anti-HuIFNβ sera with the dilutions of anti-HuIFNα and then assaying for neutralization of the test HuIFNα on GM-2504 cells (Figure 3). The neutralization curve thus obtained on the GM-2504 cells was sigmoidal and the slope of the curve was similar to that found on the FS-4 and MDBK cultures. The degree of neutralization on each cell type was determined to be inversely proportional to the degree of activity exhibited by the HuIFNα on the different cells and this was reflected by the relative positions of the neutralization curves.

During the course of these studies, it became apparent that this sensitive method also allows the detection and quantitation of antigenically distinct interferons, which are frequently present in crude IFN preparations. The biphasic neutralization pattern of the HuIFNα on the GM-2504 revealed the existence of an antigenically distinct IFN. The complete neutralization of antiviral activity at the lowest dilution (1/32) of serum, probably is due to low levels of anti-HuIFNβ antibodies, for it has been shown that certain anti-HuIFNα neutralizing sera have a population of specific anti-HuIFNβ antibodies (6). The quantity of HuIFNβ present in the HuIFNα preparation reacted with the anti-HuIFNα serum could be estimated from the HuIFNβ antiviral activity dose-response curve on GM-2504 cells, by finding the point on the curve causing 2 logs of virus inhibition. Because of the extreme sensitivity of the assay system, as little as 0.01 units/ml of HuIFNβ could be detected in the reaction mixture having a final concentration of 20 units/ml of HuIFNα. Had anti-HuIFNβ serum not been available to identify the minor interferon species, the identification of this interferon as HuIFNβ could have been based on the physicochemical and biological properties which serve to differentiate the 3 distinct HuIFN classes (α,β,γ). A conclusion could have been reached that the IFN species in the HuIFNα preparation was HuIFNβ based on the fact that the IFN had been acidified (γIFN is destroyed by low pH) and on the differential sensitivities of the 3 cell types to the non-neutralized IFN.

2. Identification and isolation of a murine interferon (MuIFN) antigenically related to HuIFNα.

Initially IFNs were characterized as being "species specific" (i.e., active only on cells of the species which produced the interferon). However, as more potent IFN preparations were produced, it was found that the IFNs from some species were active on cells of other species. In reviewing the literature, it was noticed that a number of laboratories reported that different preparations of MuIFN exerted varying degrees of antiviral activity on human cells. Being interested in both the cross-species activity of interferons and the antigenic properties of IFNs, a series of antiserum neutralization assays of both the homologous and heterologous human antiviral activities of several different murine IFNs were performed. The specific antisera used were anti-MuIFN (prepared against L-cell IFN), anti-HuIFNα, and anti-HuIFNβ. The anti-MuIFN and anti-HuIFNα sera both neutralized the MuIFN antiviral activity on human cells; however, the anti-HuIFNα serum did not neutralize the homologous (murine) antiviral activity of the MuIFN preparation. The inability of the anti-HuIFNα serum to neutralize the MuIFN activity on murine cells, suggested the possibility that the MuIFN preparation might contain two distinct IFNs, one of which exhibited heterologous human activity and was recognized by the anti-HuIFNα serum. In a series of studies, (7) a MuIFN was isolated and found to be responsible for all the heterologous antiviral activity of the unfractionated MuIFN preparation. This MuIFN interferon

was isolated by means of antibody affinity chromatography using anti-HuIFNα antibodies covalently coupled to Sepharose-4B as an immunoadsorbant.

The MuIFN activity which passed through the antibody column was devoid of any heterologous antiviral activity, whereas the MuIFN specifically bound by the column was active on murine as well as, on human and bovine cultures. The murine and human antiviral activities of this isolated subspecies of MuIFN were neutralized by anti-HuIFNα serum. The anti-MuIFN serum, which was raised against the unfractionated MuIFN, also neutralized the heterologous and homologous antiviral activities of the isolated MuIFN subspecies, however, the neutralizing titer of this antiserum for the homologous antiviral activity was 15X lower than for the antiviral activity of the unfractionated MuIFN. This indicated that the isolated MuIFN subspecies was antigenically distinct from the major MuIFN species and polyclonal antibodies raised against the whole MuIFN preparation either exhibited less affinity (reflected by the lower neutralizing titer) or possessed a distinct neutralizing antibody population specific for this MuIFN subspecies.

Experiments performed in collaboration with Dr. W.E. Stewart have determined the molecular weight (MW) of the MuIFN subspecies that is active on human cells and is neutralized by anti-HuIFNα serum (8). Molecular weight determinations by sodium-dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) showed that the unfractionated MuIFN preparations possess two MW species of 38,000 and 26,000 daltons, and all the heterologous antiviral activity was found to migrate at the leading edge of the 26,000 component. The isolated MuIFN subspecies was determined to have a MW of 20,000 daltons, whereas the major MuIFN which passed through the affinity column had an activity profile along the gel similar to that of the unfractionated MuIFN. Of considerable interest was the fact that the gel fractions containing the antiviral activities of the isolated subspecies of MuIFN showed progressively lower murine-to-human antiviral activity ratios with the faster migrating fractions. This indicated that the smaller sized molecules exhibited more human activity than the larger MuIFN components isolated by the anti-HuIFNα affinity chromatography procedure.

3. Properties of MuIFNs synthesized in the presence of tunicamycin.

The foregoing studies established that a small MW MuIFN (20K) was responsible for mediating the heterologous activities exhibited by MuIFN preparations. The finding that the smallest MW MuIFN exhibited highest [illegible] of antiviral activities on heterologous cells prompted a study to determine the effects of an inhibitor of glycosylation (tunicamycin) on IFN antiviral activities. Tunicamycin specifically inhibits the formation of the N-acetylglucosamine-lipid intermediates involved in forming the N-glycosidic linkage between core oligosaccharides and polypeptides (9, 10). Inhibitors of glycosylation have been shown previously to decrease the size and charge heterogeneities of interferons (11). We found that interferon synthesized by murine L-cells incubated with concentrations of 2.0 μg/ml of tunicamycin exhibited 4-fold less antiviral activity on homologous murine cultures, but that the heterologous activites on human cells was not reduced as compared to the interferon synthesized in the absence of the antibiotic (12). The antiviral activities of the interferons produced in the presence of tunicamycin were neutralized by specific anti-MuIFN sera to the same degree as control L-cell interferon. A 17,000 MW interferon was synthesized in the presence of tunicamycin, whereas two larger (36,000 and 26,000) interferon components were synthesized by control cultures. Isoelectric focusing of a control L-cell interferon revealed considerable molecular microheterogeneity, whereas interferon produced in the presence of tunicamycin focused as two

well-defined peaks, only one of which was neutralized by antisera raised against HuIFNα.

4. Antichlamydial actions of Interferon.

In a collaborative study with Drs. Rothermel and Byrne (Cornell Medical College) experiments were undertaken to determine the effect of interferon on chlamydial growth in vitro (manuscript in preparation). These studies were initiated to determine whether IFN exhibits an antichlamydial action, and if so, at what stage in the unique multiplication cycle of these obligate intracellular bacteria is the interferon action manifested. Murine L-cell IFN (>200 units/ml) inhibited the intracellular growth of strain 440L of C. trachomatis that causes lymphogranuloma venereum (LGV). However, MuIFN had no effect on the growth of 3 different strains of C. psittaci in mouse L-cells. The antichlamydial action on L-cells required both RNA and protein synthesis by host cells. The cellular uptake of the LGV was not affected by MuIFN. Chlamydial growth was inhibited to a similar degree when cells were pretreated with MuIFN for 18 hrs prior to infection, or up to 6 post infection. Electron microscopy was consistent with the interpretation that the antichlamydial action of IFN is caused by inhibition of successive binary divisions of the vegatative reticulate body. The inhibitory effect was found to be bacteriostatic rather than bactericidal on the LGV.

5. Induction and Initial Characterizations of Macrophage IFNs.

Studies were initiated, in collaboration with my colleague, Dr. George Spitalny, to determine the physical properties of macrophage IFN, and to investigate the effects of different MUIFNs on macrophage functions and physiology (13). Dr. Spitalny has developed the methodology to obtain confluent pure macrophage cultures derived from bone marrow (13,14).

(a). Induction of Murine Macrophage IFN.

Confluent macrophage cultures were induced with Newcastle disease virus (NDV) at different multiplicities of infection (MOI), varying concentrations of polyinosinic-polycytidylic acid ((Poly (I)-Poly (C)), or endotoxin. Both NDV and Poly (I)-Poly (C) induced interferon, however endotoxin did not. Cultures were next pretreated (primed) with varying concentrations of MuIFN for 18 hours and induced with these same IFN inducing agents. It was found that whereas priming abolished the ability of NDV to induce IFN, it greatly enhanced the levels of IFN synthesized by macrophages induced by Poly (I)-Poly (C). Moreover, it endowed the macrophage with the capacity to produce IFN in response to endotoxin.

The kinetics of IFN released from macrophages after both Poly (I)-Poly (C) and NDV showed that the Poly (I)-Poly (C) induced IFN synthesis peaked between 4-5 hours whereas, NDV induced synthesis peaked later, between 10-12 hours after initiation of induction. Under optimal conditions, NDV induction of macrophages resulted in the synthesis of 1 unit/10 cells which is at least 5x greater than the maximum synthesis elicited in L-cells, and much greater than the yield from other murine diploid cell types.

(b). pH Stabilities and Antigenicity of Macrophage IFN.

Poly (I)-Poly (C) induced macrophage interferons were analyzed as to their stabilities to low pH (2.0) antigenic properties. This IFN was found to be stable

for 5 days at pH 2.0, and was neutralized by an anti-MuIFN raised against L-cell IFN. These findings indicated that macrophage IFN was not a γIFN. Macrophage IFN also exhibited a similar degree of heterospecific antiviral activity on human cells as was observed with L-cell IFN. Antisera neutralization assays of the homologous (murine) and heterologous (human) antiviral activities of both L-cell IFN and macrophage IFN were neutralized to some degree by an anti-MuIFN serum (Table 1). The neutralizing titer of anti-HuIFNα serum for the heterologous human activities of L-cell and macrophage IFN was similar. From these studies, it was tentatively concluded that macrophage IFN was antigenically indistinguishable from IFN produced by L-cells.

(c). Physicochemical Properties of Macrophage IFN.

(1). M.W. determinations. Samples of IFNs produced by macrophages, L-cells, and murine embryo fibroblasts were made to 0.1% SDS and 0.1% mercaptoethanol (ME) and boiled for 1 minute and then analyzed by SDS-Page to determine the MW of each IFN (Figure 4). The activity profiles along the gels clearly show that the L-cell and mouse embryo fibroblast IFNs migrated as two defined peaks of antiviral activity corresponding to M.W.s of 36,000 and 26,000 daltons. The macrophage IFN showed one peak of activity at 36,000. However, it should be pointed out that prior to electrophoresis, 90% of the original macrophage IFN was abolished by the SDS and boiling procedure. The activities of the two other IFNs remained relatively stable to this treatment.

A study was done to determine the thermal stabilities of L-cell and macrophage IFNs, and a γIFN preparation obtained by inducing murine spleen cultures with phytohemagglutinin (PHA). Samples of each interferon were made to 0.1% SDS in the presence of absence of ME and then boiled at 100°C/1 min. The effect of these treatments on the anti-viral activities of each IFN are presented in Table 2. All the test IFNs, not treated with SDS, lost all of their activities after boiling. In the presence of SDS alone, all of the macrophage IFN and γIFN activities were destroyed whereas 75% of the L-cell IFN was abolished. Under reducing conditions, 12% of the macrophage IFN and 75% of the L-cell IFN activities remained after boiling. The γIFN activity was abolished under reducing conditions. Similar results were also obtained if the 3 IFNs were treated with SDS, under reducing or non-reducing conditions, and then incubated at 37°C/hr. The findings of these studies clearly demonstrated that SDS, under reducing or non-reducing conditions, destroys the majority of macrophage IFN and all of the γIFN antiviral activity. Thus, meaningful MW determinations had to be performed by a means other than SDS-PAGE.

The MWs of the three murine IFNs were determined by molecular seiving chromatography on a Biogel P-100 column. Prior to chromatography, considerable effort was placed on finding a buffer in which the γIFN was stable. It was found that all three IFNs were very stable in 0.05 M Na citrate buffer (pH 6.0). The elution profile of the γ, L-cell, and macrophage IFNs are presented in Figure 5. Ovalbumin (43,000) and cytochrome C (13,000) were added to each sample prior to chromatography to serve as MW markers, even though the column had been calibrated previously. The γIFN was determined to have a MW in the range of 35,000-40,000 and appeared to elute as a single peak. The murine L-cell IFN activity profile was heterodisperse, running from 20,000-40,000 daltons. Interestingly, the macrophage IFN appeared to possess a major peak of activity at 45,000 associated with a smaller shoulder of activity at 35,000. No macrophage IFN activity was observed in the lower MW region (20-30K) evident in the L-cell IFN elution profile. The recoveries of applied interferons were routinely in the range of 50-100%. Thus, the combined results of the column

chromatography and the SDS-PAGE studies indicated that while L-cell IFN consists of two MW interferons (36K and 26K), the macrophage IFN preparation appeared to have two peaks of activity in the MW range 35,000-45,000.

(d). Isolation of Macrophage α and β IFNs. Macrophage IFN exhibited the same degree of cross-species activity on human cultures as L-cell IFN. Since it has been established that the L-cell IFN component responsible for mediating the antiviral activity on human cells is associated with the smaller MW component of L-cell IFN, it was deemed important to isolate the macrophage IFN responsible for the antiviral activity on human cells and to determine its MW. NDV induced macrophage IFN was passed through an anti-HuIFNα antibody column, and the specifically retained and unretained fractions were assayed on murine and human cells (Table 3). Approximately 25% of the applied macrophage antiviral activity was specifically bound (retained) by the anti-HuIFNα column and only this fraction exhibited antiviral activity on human cells. The results of antigenic analysis of the retained and unretained macrophage IFNs as well as those of L-cell IFNs homologous antiviral activities are presented in Table 4. An antiserum raised against MuIFN from L-cells (anti-MuIFN) neutralized the homologous antiviral activities of the various fractions, however, the neutralizing titer for the specifically retained activities of both the L-cell and macrophage IFNs were lower than for the unretained activities. An anti-HuIFNα serum neutralized the antiviral activities of only the retained fractions of both the L-cell and macrophage IFNs to the same degree. Anti-HuIFNβ did not neutralize the activities of any murine IFN fraction.

Having isolated and characterized the antigenic properties of two IFN components of macrophage IFN, we next determined the MW of each fraction separated by antibody affinity chromatography on an anti-HuIFNα immunoadsorbant column by means of a Biogel P-100 column (Figure 6). The antiviral activity elution profile of the unretained activity shows the major peak of activity eluting at 43,000 and shows the second peak of activity following at 35,000 was reduced when compared to the activity present in the unfractionated IFN (applied). The retained activity eluted in a region corresponding to a MW of 35,000 with very little activity at 43,000. It should be pointed out that in the activity profiles for both the retained and unretained macrophage IFNs, there are peaks of activity eluting in a higher MW region which are not present in the unfractionated (applied) IFN. It is possible that these peaks of IFN may represent aggregates formed during the concentration of the two antibody affinity isolated fractions prior to the chromatographic procedure.

Summary of Importance of These Findings.

We have developed a highly sensitized, objective antibody neutralization assay for IFN which measures IFN neutralization by virus yield, and allows the detection and quantitation of antigenically distinct IFNs not neutralized by the specific antibody. This means of IFN neutralization will not only prove valuable in characterizing the IFN components in a given IFN preparation, but will also enable studies to determine the degree to which other biological activities are neutralized. Differences in the slopes of the antibody neutralization curves for different IFN activities or a relationship which is not inversely proportional between the antibody neutralizing titer for an interferon activity and the quantity of interferon (antigenic mass reacted as quantitated by relative antiviral activity), could indicate the possibility that distinct IFNs within an IFN class may differ in their abilities to exert different biological actions.

The combined work of Yamamoto (15), Taira (16) and the work performed in this laboratory (7) has demonstrated that MuIFNs (type I; i.e. acid stable) consist of a mixture of distinct α and β MuIFNs which are structurally related to the respective HuIFN classes. Thus, it should be feasible to determine the possible function of each IFN class (α,β,γ) in various murine models of infectious and malignant diseases.

Cultured murine macrophage can be stimulated to produce a very high level of IFN. Macrophage IFN contains two IFN components which are antigenically related to α and β L-cell IFN, however, the macrophage IFNs differ in size and stabilities in SDS when compared to other MuIFNs.

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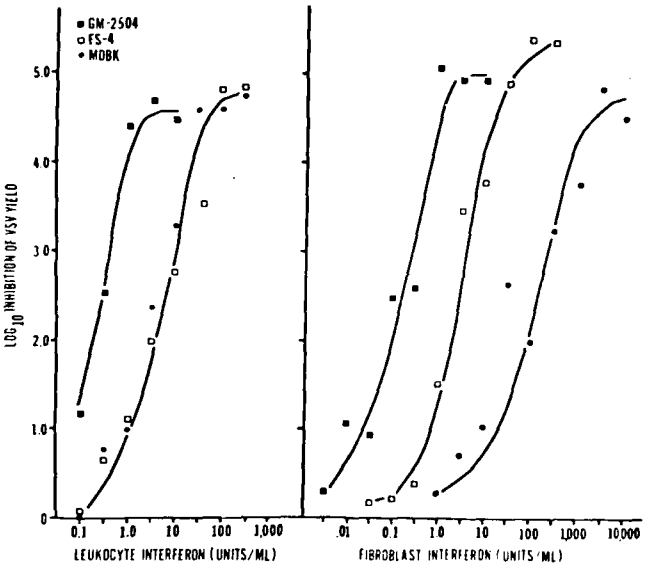


Fig. 1. Dose-response curves of human leukocyte and fibroblast interferons after an 18 hour incubation on human GM-2504, human FS-4, and bovine MDBK cultures. Virus yields were determined after a single cycle of VSV replication

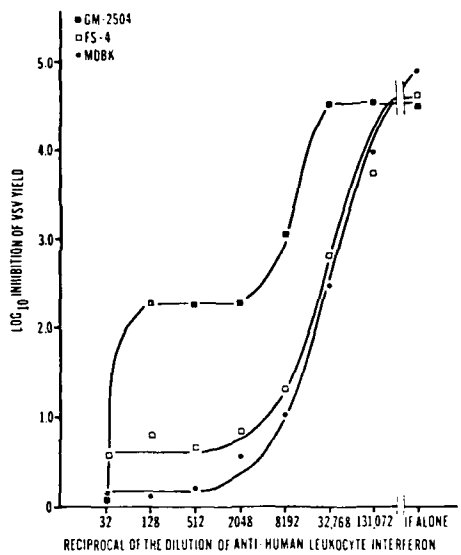


Fig. 2. Anti-Le interferon antibody neutralization curves for Le interferon activities on human GM-2504, human FS-4, and bovine MDBK cultures. A constant quantity of human Le interferon [final concentration of 20 international interferon (IF) reference units as determined on FS-4 cells] was reacted with an equal volume of each dilution of antibody and placed on the cultures for 18 hours. Virus yields were determined after a single cycle of VSV replication

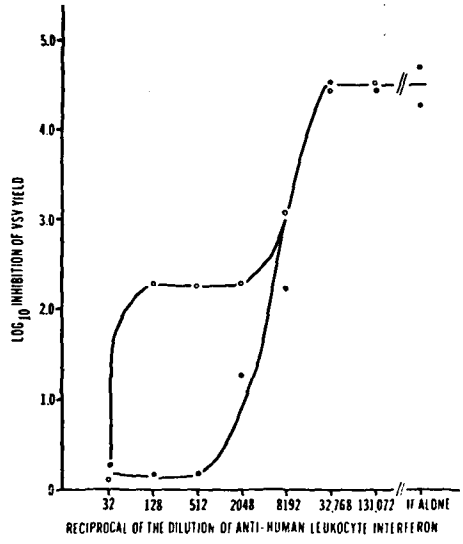


Fig. 3. Antibody neutralization curves of Le interferon activity on human GM-2504 cultures by anti-Le interferon antibody alone (o) or in the presence of a constant quantity of high-titered anti-F interferon serum (•)

Figure 4

Molecular Weight Determinations of Different Murine Interferons by SDS-PAGE

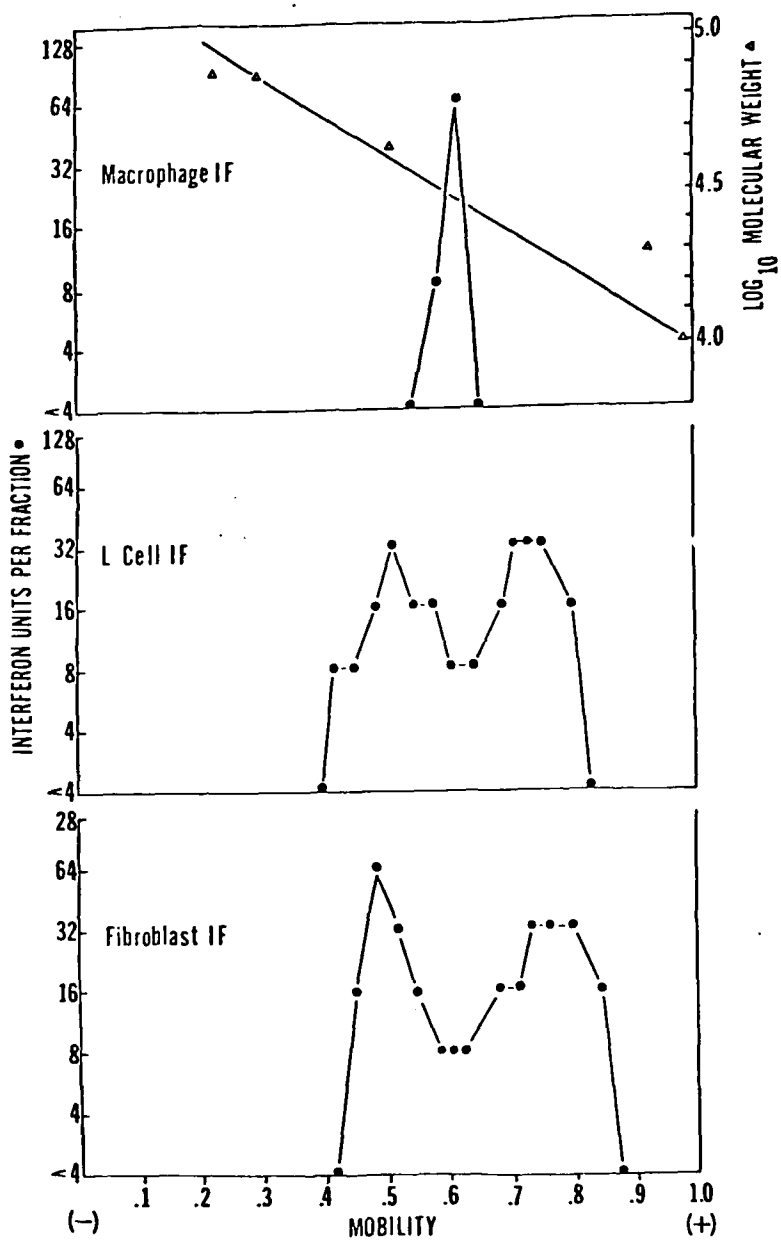
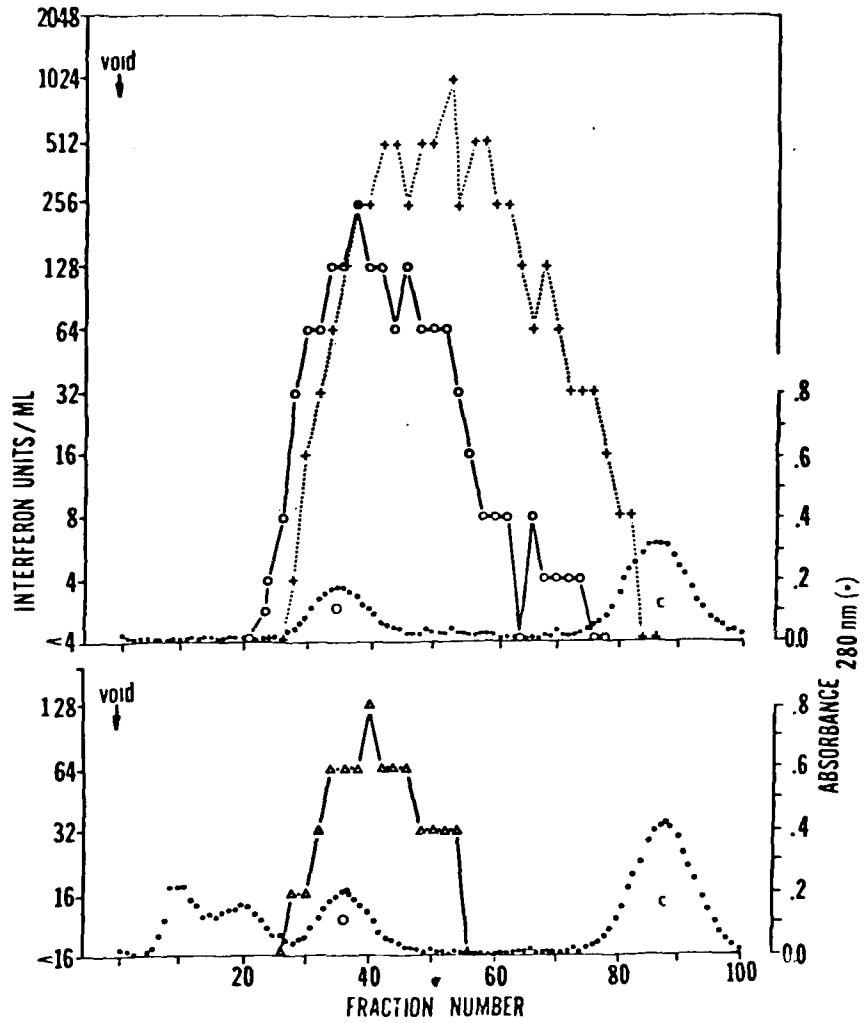


Figure 5

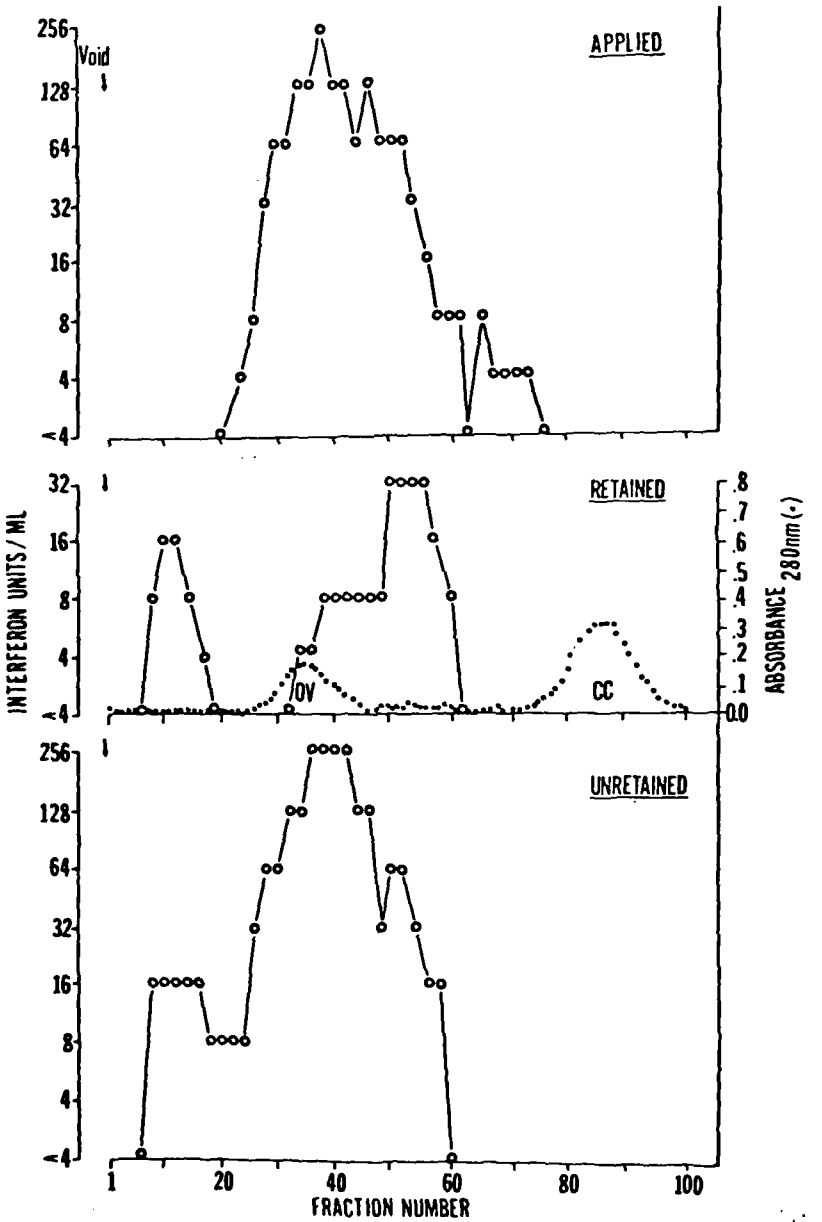
Elution Profiles of Murine L-Cell (+), Macrophage (o), and Spleen cell (Δ) IFNs from a Biogel P-100 Column



O, ovalbumin MW marker
C, cytochrome C MW marker

Figure 6

Biogel P-100 Column Elution Profiles of Macrophage IFN Fractions Isolated by an Anti-HuIFN$_\alpha$ Affinity Chromatography Column



OV ovalbumin MW marker
CC cytochrome C MW marker

Table 1

Antiserum Neutralization Assays of the Homologous and Heterologous Antiviral Activities of Different Interferons

Cell source of interferon	Neutralization titer[a] of			
	Anti-murine L cell IF on		Anti-human α IF on	
	Murine (L929B)	Human (GM2504)	Murine (L929B)	Human (GM2504)
Murine L cells	1600	20	<25	200
Murine macrophages	800	50	<25	200
Human leukocytes	<25	<25	280	14,746

[a]Reciprocal of the dilution of antiserum which when mixed with an equal volume of interferon (having 20 units of antiviral activity on the respective assay cell) neutralizes greater than 50% of the antiviral activity.

Table 2

Comparison of the Thermal Stabilities of Murine Interferons in the Presence of SDS under Reducing and Nonreducing Conditions.

		Interferon (Units/ml)		
Treatment	Incubation	L Cell[a]	Macrophage[a]	Spleen[b]
none	none	1024	512	256
none	100°C/1 minute	<16	<16	<16
SDS	100°C/1 minute	256	<16	<16
SDS+ mercaptoethanol	100°C/1 minute	768	64	<16

a. L-cell and Macrophage IFNs induced by Poly(I)-Poly(C)

b. γIFN; induced by PHA (5μg/ml) stimulation of spleen cell cultures

Table 3

Homologous and Heterologous Antiviral Activities of Murine Macrophage Interferon Components Separated by Affinity for Anti-Human Leukocyte Interferon Antibody

	Interferon Activity on cells		
Fraction	Murine (L929B)	Human (GM2504)	Murine/Human
Applied[a]	6144	144	43
Unretained	5515	<4	>1300
Retained[b]	1454	45	32

[a]Murine macrophage interferon applied in phosphate-buffered saline (pH 7.4) to a column of anti-human Le interferon antibody covalently coupled to Sepharose 4B.

[b]Antiviral activity specifically retained by the antibody column and eluted with 0.1 M acetic acid containing 0.5 M NaCl pH 3.1.

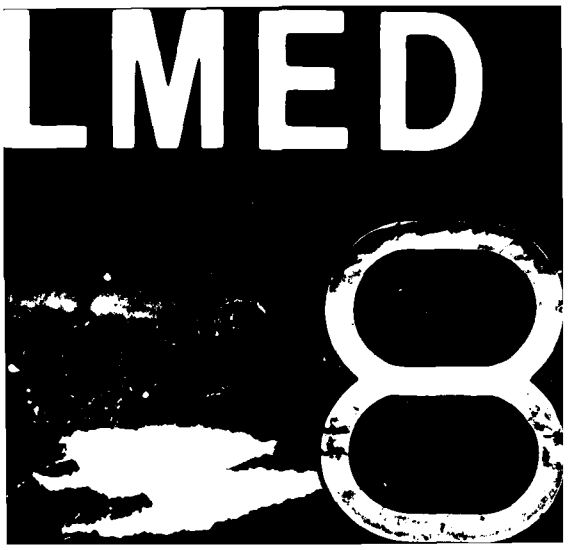
Table 4

Antigenic Properties of Murine L Cell and Macrophage Interferons Isolated by Affinity Chromatography on an Anti-HuIFN$_\alpha$ Antibody Column

Source of interferon	Column fraction	Neutralization titer[c] on murine L Cells by		
		Anti-MuIFN	Anti-HuIFN$_\alpha$	Anti-HuIFNβ
L cells	Applied	1600	<25	<25
	Unretained	1600	<25	<25
	Retained	400	50	<25
Macrophages	Applied	400	<25	<25
	Unretained	400	<25	<25
	Retained	200	50	<25

[a]Rabbit anti-HuIFN$_\alpha$

[c]Reciprocal of the dilution of antiserum which when mixed with an equal volume of interferon having 20 units/ml neutralizes 50% of the protective effect as judged by virus cytopathic effect.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